bio-protocol Preprint

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RNAscope in situ hybridization

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An abbreviated version of this protocol was published in eLIFE in Sep 2019

A distinct cardiopharyngeal mesoderm genetic hierarchy establishes antero-posterior patterning of esophagus striated muscle

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Detailed protocol

RNAscope protocol (Tajbakhsh Lab)

Embryo processing

1. Fix embryos on 4% PFA at 4C with rocking overnight (if possible cut the embryo head or at trunk level to allow better penetration of the PFA)
2. Rinse twice with PBS and wash for few hours at 4C with rocking
3. Equilibrate embryos in Sucrose 30% in PBS
4. Embedd embryos in OCT and store them in sealed bags at -80
5. Section blocks at 14-18um. Keep slides at -80 and use ASAP

RNAscope pretreatments

6. Bake slides 30min 55C in a slide rack inside a staining dish
7. Wash slides in PBS 5min (move the slide rack up-down 5 times) to remove OCT. I use commercial Invitrogen PBS for this
8. Flick slides one by one to remove excess of liquid and lay down in a wet chamber. Apply RNAscope H2O2 reagent 10 min RT
    - During this incubation time or earlier, put to heat up retrieval buffer in a hot plate set to 300C (for 400ml of 1x retrieval buffer = 40ml stock + 360ml H20). Control the temperature to achieve 95C.
    - Put the oven to heat to 40C containing the wet RNAscope slide chamber (including filter paper and H20)
9. Wash slides in distilled H20 in a slide rack inside a staining dish (move the slide rack up-down 5 times). Repeat with fresh distilled H20.
10. Put the slide rack into the beaker with retrieval buffer at 95C. For “fragile” tissues that are not postfixed do retrieval for 2-5 min
11. Remove slide rack and immediately transfer to a staining dish containing distilled H2O. Rinse for 15sec
12. Transfer the slide rack to a staining dish containing Ethanol 100% for 3min
13. Bake slides at 55C for 5min
14. Check sections under a loupe and select the ones that are going to be stained. Apply hydrophobic pen (Immedge TM). Let dry 5min RT
15. Optional: Quick rinse of slides in PBS 0.01% Tween (this helps the probes to “extend” properly, especially if hydrophobic barrier was drawn close to sections
16. Lay down slides on the RNAscope slide chamber and add Protease Plus drops. For “fragile” tissues that are not postfixed do PK treatment for 15 min
    - During this incubation time prepare probes: Warm up probes 10min at 40C
    - If C1 probes, they are ready to use
    - If C2 probes: dilute 1/50 in Probe diluent
    - If C1 + C2 probes: dilute C2 1/50 in C1 probe. (15 drops = 300ul)
17. Flick slides one by one to remove excess of liquid and transfer to a staining dish containing distilled H2O (slide rack up-down 5 times)
18. Optional: Quick rinse of slides in PBS 0.01% Tween (this helps the probes to “extend” properly, especially if hydrophobic barrier was drawn close to sections

Hybridization of probes

19. Flick slides one by one to remove excess of liquid and ay down slides on the RNAscope slide chamber. Add probes and incubate for 2h 40C
    - During this time: put the kit to equilibrate at RT. I do all subsequent steps under a hood (some chemicals in the kit are carcinogens)
    - Prepare wash buffer (for 1L of wash buffer = 20ml 50X + 980ml H20)

Note: All subsequent steps (wash and incubation with reagents) are done like this: Wash slides in 1X Wash buffer in a staining dish 2min RT (move slide rack up-down 5 times). Repeat with fresh Wash buffer. Flick slides one by one to remove excess of liquid and lay down slides on the RNAscope slide chamber. Add drops of xx reagent and incubate xx time.

FOR COLORIMETRIC KIT 2.5HD Red on channel 1 → fast red can be seen by TL or as red fluorescence in Cy3 channel

20. Wash 2x 2 min RT
21. AMP1 30 min 40C
22. Wash 2x 2 min RT
23. AMP2 15 min 40C

23. AMP2 15 min 40C
24. Wash 2x 2 min RT
25. AMP3 30 min 40C
26. Wash 2x 2 min RT
27. AMP4 15 min 40C
28. Wash 2x 2 min RT
29. AMP5 30 min RT
30. Wash 2x 2 min RT
31. AMP6 15 min RT
    - During this step prepare Fast Red solution: 2ul Red_B per 120 Red_A. Mix well.
32. Wash 2x 2 min RT
33. Apply Fast Red solution 10 min RT (or 5 min if staining too strong). 50-100ul per section.
34. Flick slides to remove Fast red in a Falcon tube (Fast Red is very toxic so the Falcon tube should be discarded in the chemical waste) and then immediately wash slides with Tap water on a staining dish. Rinse again with fresh Tap water.
35. Wash with PBS and mount or proceed to immunostaining/IHC if required.

NOTE: We realized for some antibodies immunostaining does not work after Fast Red staining.
36. In those cases, after AMP6 step, wash slides with PBS 2x 5min RT, block with blocking buffer (15% Goat Serum, 1.5% BSA, 0.5% triton in PBS) 30 mir RT, add primary antibodies ON. Wash 2x 5 min RT with PBST (0.1% tween) and add secondary antibodies and Hoechst as for normal immunostaining (be careful with channels used so not to overlap with RNAscope dyes). Wash 2x 5 min RT with PBST (0.1% tween)
37. Wash 2x 5 min with 1X RNAscope Wash buffer
38. Detect probes with steps 33-35.

FOR FLUORESCENT KIT V2
From step 19 proceed as follows:
39. Wash 2x 2 min RT
40. AMP1 30 min 40C
41. Wash 2x 2 min RT
42. AMP2 30 min 40C
43. Wash 2x 2 min RT
44. AMP3 15 min 40C
45. Wash 2x 2 min RT
46. If C1 probe: HRP_C1 15 min 40C
    - During this incubation time prepare Opal dilutions (see below)
47. Wash 2x 2 min RT
48. Apply Opal dye diluted in TSA buffer (50-100ul/section) 1/1500 for Opal 520 and Opal 570. If Opal 650 is used for C1 use at 1/1000. Incubate 30 min 40C
49. Wash 2x 2 min RT
50. HRP blocker 15 min 40C
51. Wash 2x 2 min RT
If using a single C1 probe stop here, otherwise, proceed to detection of C2 and C3 as above (use HRP_C2/C3 instead).
52. If performing immunostaining: wash with PBS 2x 5min RT, block with blocking buffer (15% Goat Serum, 1.5% BSA, 0.5% triton in PBS) 30 min RT, add primary antibodies ON. Wash 2x 5 min RT with PBST and add secondary antibodies and Hoechst as for normal immunostaining (be careful with channels used so not to overlap with RNAscope dyes). Note: not all antibodies work after the pretreatments of the RNAscope protocol. Mount slides in 75% Glycerol in PBS

How to cite:(Readers should cite both the Bio-protocol preprint and the original research article where this protocol was used)

1. Comai, G. (2020). RNAscope in situ hybridization. Bio-protocol Preprint. bio-protocol.org/prep505.
2. Comai, G., Heude, E., Mella, S., Paisant, S., Pala, F., Gallardo, M., Langa, F., Kardon, G., Gopalakrishnan, S. and Tajbakhsh, S.(2019). A distinct cardiopharyngeal mesoderm genetic hierarchy establishes antero-posterior patterning of esophagus striated muscle. eLIFE. DOI: 10.7554/eLife.47460